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14. ABSTRACT Various antiangiogenic strategies have proven effective in preclinical tumor models, either as single agents or combined with radiation (RT). The current work aimed to evaluate whether treatment sequencing critically impacts tumor pathophysiological and therapeutic response. Using human prostate tumor models, axitinib, an inhibitor of VEGF receptors, was administered either before or after each daily RT fraction, and pathophysiological changes were monitored. Tumor growth inhibition was equivalent following the two combination schedules. Similar reductions in blood vessel counts were observed with each, tumor hypoxia increased, and pericytes progressively dissociated. These studies illustrate a clear advantage to combining axitinib with fractionated therapy, but argue against an acute radiosensitization or radioprotection of either the tumor cells or tumor vasculature. Instead, post- and pre-RT drug administration serve equally well in supplementing radiotherapeutic response.					
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Introduction:

Since some funds remained following the original grant end date, we continued working on our two prostate xenograft lines to further extend our pathophysiological studies for publication. This addendum report covers the research conducted during the no-cost extension, September 10, 2007 – December 10, 2007, and incorporates our latest data into the previous reported plots. We are in the final stages of writing these experiments up for submission to Radiation Research, and the results are summarized herein.

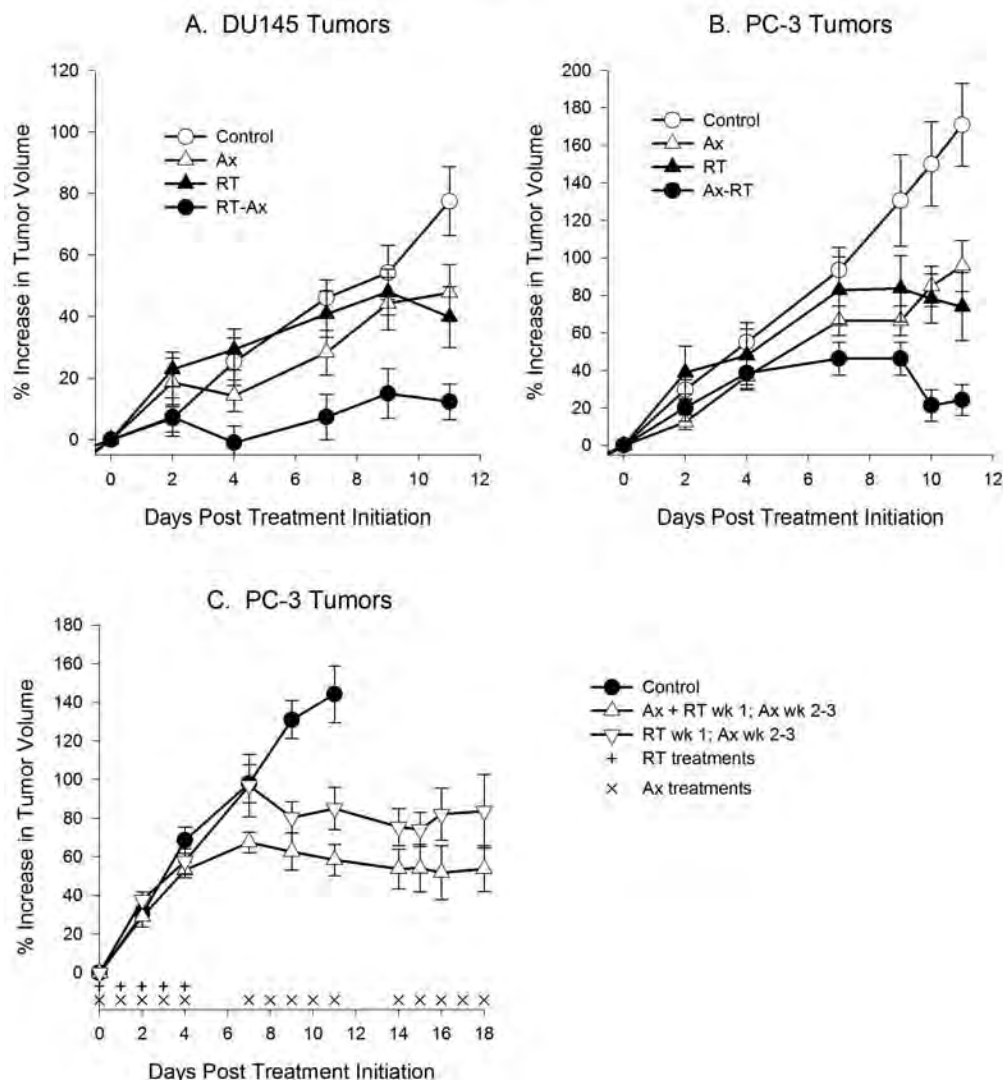
The current work was undertaken to evaluate the importance of altering the daily sequencing of therapies, specifically combining fractionated RT with axitinib (AG-013736, Pfizer Global Research and Development), a receptor tyrosine kinase inhibitor that predominantly inhibits vascular endothelial growth factor receptors (VEGFRs). Axitinib was administered either 1 hr pre- or 1 hr post- each 2 Gy RT fraction. Over short times, at least two opposing mechanisms could conceivably play a role. Since inhibitors of VEGFRs have been shown to sensitize endothelial cells to RT (ref), pre-RT axitinib dosing could lead to an enhancement of radioresponse. Alternatively, since axitinib has also been shown to significantly increase tumor hypoxia, at least over periods of days-weeks¹, pre-RT dosing could also acutely decrease radioresponse. To better comprehend the underlying pathophysiological mechanisms, temporal alterations in tumor vascular spacing, pericyte and basement membrane coverage, and hypoxia were quantified in two prostate tumor models during the alternate schedules. Although combination therapy produced substantial alterations in tumor pathophysiology, modifications in the daily order of therapy were not significant.

Body:

Tumor progression is significantly inhibited by single and combined treatments as well as maintenance axitinib. In order to gauge the additive benefits of combined therapy, suitable doses of axitinib or RT were chosen, based on earlier results¹, in order to slow but not entirely stop tumor progression. Single or combined treatments were given over a period of 2-3 weeks, and tumor volumes were measured three times per week as shown in Figures 1A-C for DU145 and PC-3 prostate carcinoma xenografts. For each tumor model, the effects of either axitinib or RT on tumor growth suppression were similar (Figures 1A-B). By day 11, the % increases in tumor volume following either monotherapy were significantly less than for vehicle-treated controls, and the % increases following the combination were significantly smaller than for single treatments. Figure 1C contrasts two additional schedules: one week of either combination treatment or RT, followed by maintenance therapy with axitinib alone for an additional two weeks. By day 7, tumor volumes were significantly reduced for the combination treated tumors compared to the controls ($p = 0.038$), but not significantly different from those treated with RT alone. Over the following two weeks of axitinib maintenance therapy, tumor volumes were not significantly different between schedules, and neither of the treated groups increased in size.

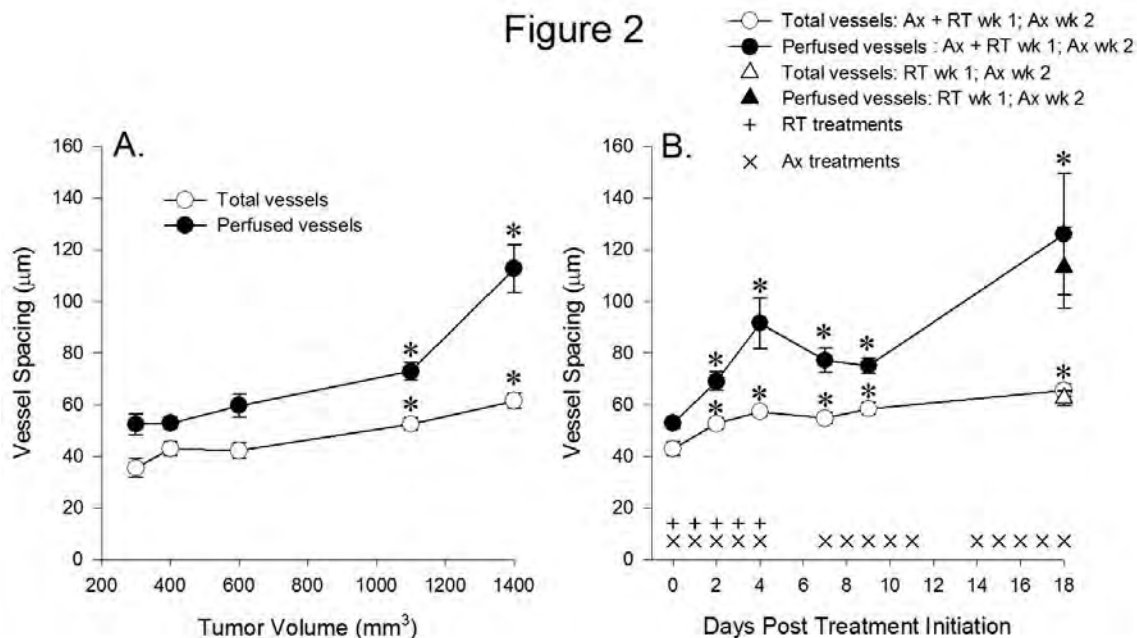
Total and perfused vessel spacing increases progressively with tumor growth in controls and perfused spacing is further compromised by

Figure 1



combination treatment. We next monitored the effects of tumor growth on vessel spacing in untreated PC-3 tumors (Figure 2A) as well as the temporal changes following combination therapy (Figure 2B). Up to a volume of $\sim 600 \text{ mm}^3$, neither total nor perfused vessel spacing varied significantly for controls, but by $\sim 1100 \text{ mm}^3$ both had significantly increased (Figure 1A). At 1400 mm^3 , perfused spacing was also markedly increased compared to the 1100 mm^3 volume.

Tumors summarized in Figure 2B were treated with the same two treatment schedules shown in Figure 1C: the first week included either RT alone or the combination of axitinib plus RT, and the following two weeks included only axitinib maintenance therapy. At the end of the first four days of combination treatment, both total and perfused vessel spacing had significantly increased (Figure 2B), despite the fact that treated tumor volumes were no different from



untreated controls at this timepoint (Figure 1C). Over the following two-week course of axitinib alone, perfused spacing remained high and, in select tumors, increased markedly by day 18 (note the large standard deviation in Figure 2B). Although perfused vessel spacing was much higher than in untreated tumors at comparable volumes of 700-900 mm³, total spacing was equivalent (Figure 2A). For the schedule beginning with RT alone, total and perfused vessel spacings following 2 weeks of maintenance therapy were equal to those of tumors initially subjected to combination therapy (Figure 2B).

Altered order of treatment does not modify tumor growth or vessel spacing. In order to determine whether axitinib produced acute alterations in tumor oxygenation, and therefore radiosensitivity, this agent was administered either 1 hr before or 1 hr following each 2 Gy fraction of RT for approximately two weeks. The alternate schedules suppressed tumor progression almost identically for both the PC-3 (Figure 3a) and DU145 (Figure 3B) tumors. Figures 4A and 4B present corresponding alterations in vessel spacing at days 0, 4, and 11 for PC-3 and days 0, 7, and 11 for DU145. For PC-3, both perfused and total vessel spacing significantly increased with increasing treatment time at almost all timepoints, compared to pretreatment controls. For DU145, perfused vessel spacing generally increased with treatment, but response was quite variable at day 11 for the pre-RT axitinib schedule. No significant schedule-dependent differences in either total or perfused vessel spacing were found at any timepoints with either tumor model.

Altered order of treatment has minimal effects on pericyte and basement membrane dissociation or hypoxia. As shown in Figure 5A, dissociation of PDGFRβ+ pericytes from blood vessels increased progressively for the axitinib pre-RT treatments. For the post-RT axitinib treatments, dissociation increased more gradually and was not significantly higher than

Figure 3

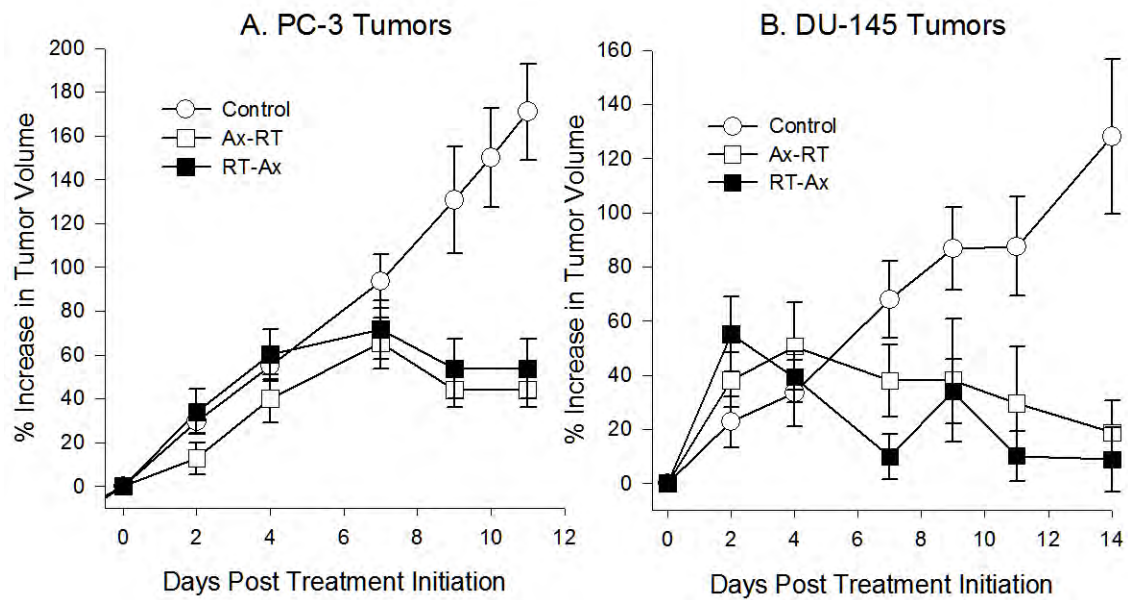
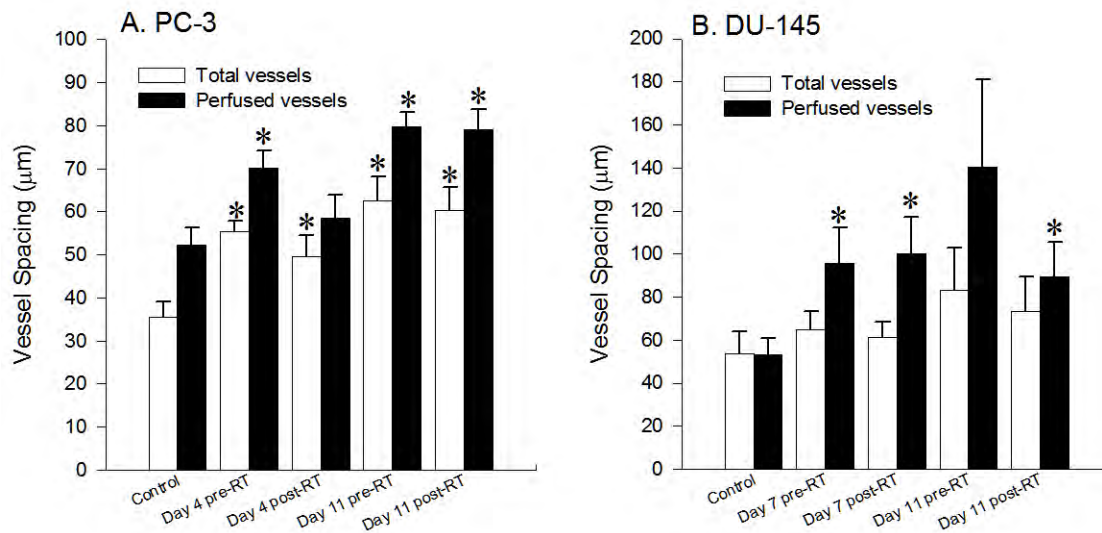
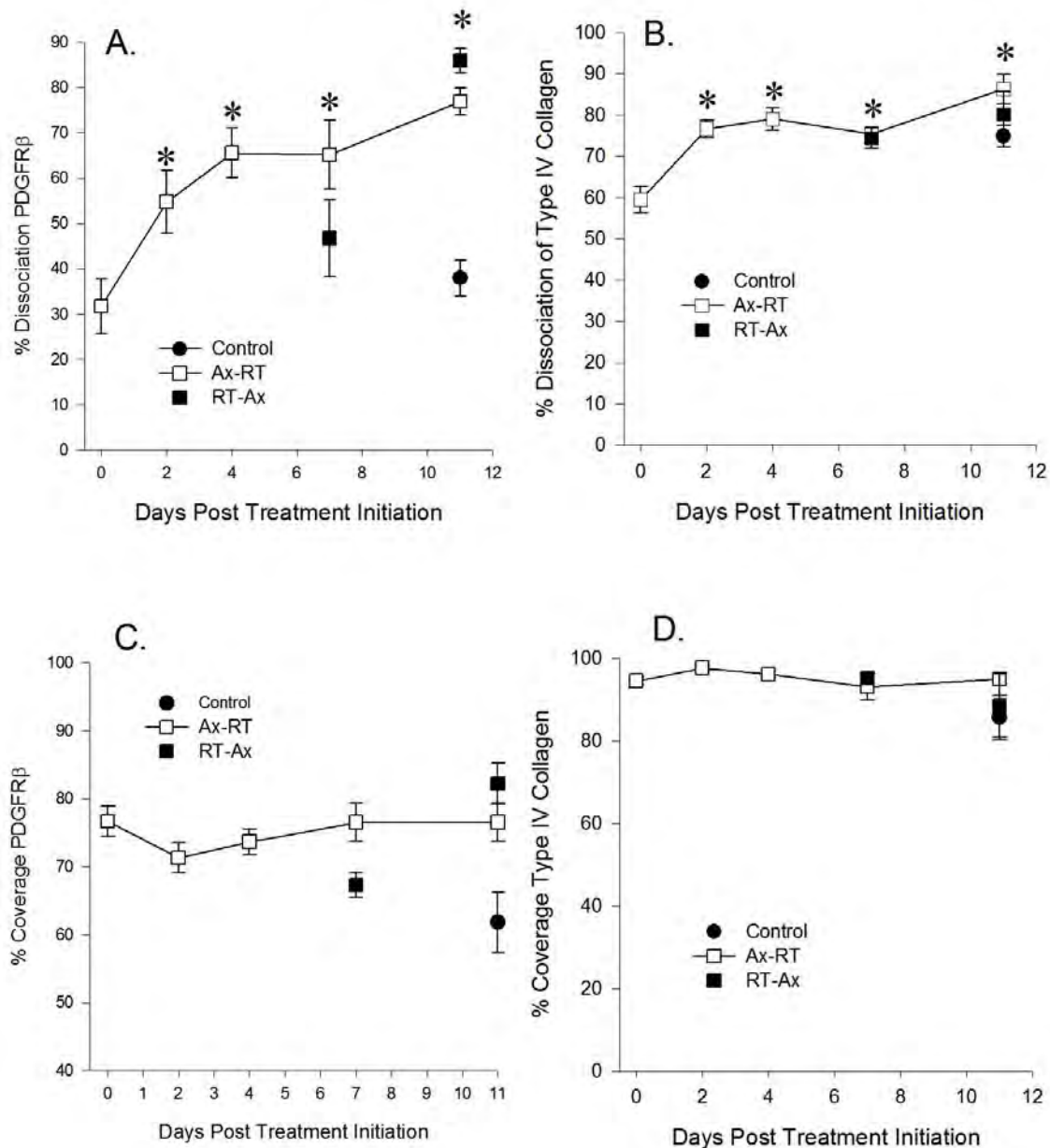


Figure 4



pretreatment controls until day 11, at which time its level approached that of the pre-RT scheduling. Large volume controls are also included in Figure 5A to illustrate the effect of tumor volume on pericyte dissociation. No significant differences were found between small and large untreated tumors.

Figure 5 - DU145

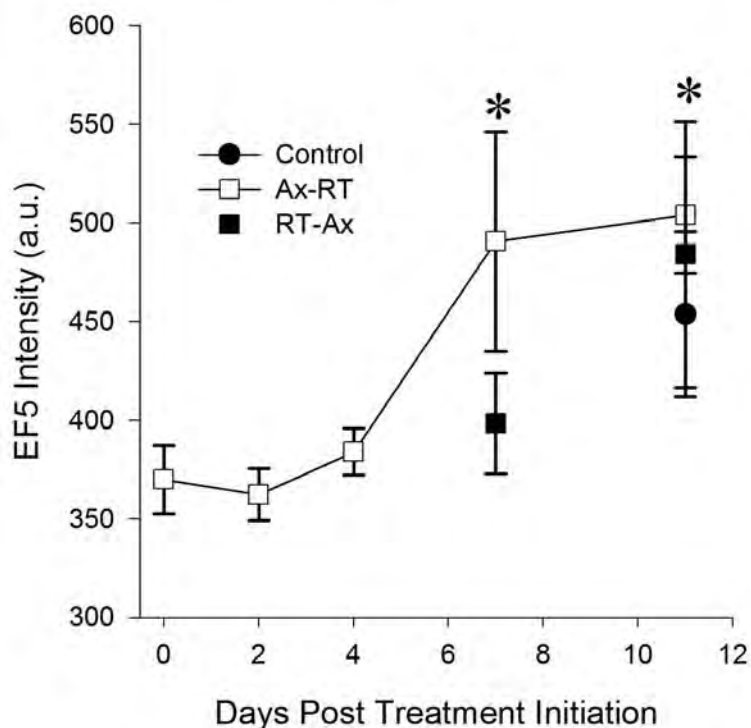


Dissociation of type-IV collagen (Figure 5B) is reflective of the dissolution of endothelial cells from their surrounding basement membrane. Both pre- and post-RT schedules resulted in significant increases in what have been sometimes termed “basement membrane ghosts”², but with no significant differences between schedules. For the type-IV collagen, dissociation in untreated tumors also increased significantly with growth. Percentages of vessels covered with either type-IV collagen or PDGFRβ were generally unchanged in relation to pre-treated controls at all timepoints (Figures 5C and 5D), except at day 7 for the post-RT schedule where PDGFRβ was somewhat reduced.

Similarly to the dissociation results, PDGFR β coverage for large untreated tumors was reduced in relation to treated tumors for either schedule, perhaps reflective of a transition to a more mature pericyte phenotype with tumor growth.

Hypoxia in the DU145 tumors, as determined using EF5 hypoxic marker binding, remained constant over the first four days of treatment but increased significantly at day 7 for the pre-RT schedule (Figure 6). Although hypoxia increased at a slower pace following the post-RT schedule, overall levels were not significantly different at either the 7 or 11 day timepoints. By day 11, hypoxia levels following either schedule were also essentially equal to the much larger volume controls.

Figure 6 - DU145



Key Research Accomplishments

- These studies demonstrate a clear advantage in combining Axitinib with fractionated RT.
- Combination treatment leads to decreased tumor growth, reduced total and functional vascular counts, increased tumor hypoxia (due primarily to the Axitinib), and enhanced pericyte and basement membrane dissociation (suggesting a breakdown of vascular integrity).
- Alternative daily sequencing of Axitinib administration and RT (1 hr pre-versus 1 hr post-) had no significant effects on tumor growth rate, vessel spacing, or hypoxia, suggesting that this agent does not acutely sensitize endothelial or tumor cells to RT.
- Maintenance therapy with Axitinib alone effectively suppressed tumor growth for up to two weeks, regardless of whether the 1st wk of treatment was RT or the combination.

Reportable Outcomes:

Abstracts and Presentations:

- 1) 2008 Radiation Research Meeting, Boston, MA. Scheduling of Axitinib in relation to radiotherapy does not impact tumor growth inhibition or pathophysiological response. BM Fenton and SF Paoni.

Personnel supported:

- 1) Bruce Fenton, PhD
- 2) Scott Paoni, MS

Conclusions:

Given the complex mix of interdependent and opposing pathophysiological effects, it is perhaps not surprising that prior studies investigating the effects of treatment scheduling have been somewhat contradictory. Although multiple studies have demonstrated clear benefits when combining RT with antiangiogenic approaches (see³ for review), negative results have also been reported⁴, and scheduling of treatments has been arbitrary. The precise effects of alternative combination RT/antiangiogenic schedules on therapeutic response have only been investigated in a handful of studies, each with different VEGFR or PDGFR inhibitors. SU11657 was found to be more effective when given one day before a single dose of 7.5 Gy than one day after, a result attributed to a possible normalization of tumor vasculature⁵. In contrast, no differences were noted when SU5416 or SU6666 was administered at either 30 min before or 30 min after each 2 Gy RT fraction, which was interpreted to suggest that significant changes in tumor oxygenation must not have occurred over this time period⁶. Williams et al.⁷ administered ZD6474 at a somewhat similar schedule of either 2 hr pre- or 30 min post- 2 Gy RT fractions. Here, the post-RT drug administration was much more effective than the pre-RT in producing growth delay, which was attributed to a drug-induced reduction in tumor blood flow for the pre-RT schedule. Since each of these studies utilized different drugs and tumor models, it should perhaps not be surprising that optimal scheduling could also vary.

We previously characterized tumor pathophysiological response following 1-3 wk regimens of 2 Gy fractionated RT, 25 mg/kg/day axitinib, or the combination¹. At weekly timepoints, both axitinib and the combination significantly reduced perfused blood vessel counts, while increasing tumor hypoxia. Endothelial apoptosis also increased for the combination regime. These results argue against a treatment-induced functional normalization of the tumor vasculature at the weekly timepoints, but earlier more acute changes in tumor oxygenation could possibly have been overlooked.

The current investigation was designed to extend these studies in two ways. First, tumors were frozen at days 2, 4, and 7 days following treatment initiation to better characterize early response. Second, two alternative combination treatment schedules were contrasted in terms of tumor pathophysiological response and overall growth inhibition. Since the prior work demonstrated a progressive increase in tumor hypoxia in response to axitinib over the first week¹, we anticipated that combination therapies scheduled to begin with axitinib alone would potentially compromise subsequent RT. We

instead compared acute sequencing alterations, specifically 1 hr pre- versus 1 hr post-RT administration of axitinib. Differences in response to these schedules is presumably reflective of both acute modifications in oxygen delivery and axitinib-induced endothelial cell radiosensitization.

At the suboptimal doses selected, both fractionated RT and axitinib somewhat slowed tumor progression in both the DU145 and PC-3 tumor models. In each case, the combination therapy produced a pronounced and sustained growth inhibition. As has been previously documented, total and perfused vessel spacing increased substantially with tumor growth for untreated tumors, and in the absence of tumor growth for tumors treated with combination therapy. In a separate experiment, sustained tumor dormancy was also observed following 2 wks of axitinib maintenance therapy, regardless of whether the initial treatment was RT alone or the combination. This agrees with previous studies showing extended tumor control when SU11248 maintenance therapy followed either RT, drug alone, or the combination⁸. Although tumor volume remained unchanged over the entire course of maintenance therapy, perfused vessel spacing progressively increased to levels equal to those of large volume untreated controls. The initial increase in vessel spacing over days 1-4 could reflect a pruning of redundant, less mature vessels in the treated tumors, followed by a continued increase over days 9-18, as the tumor cells progressively outgrow their vasculature. In control tumors, tumor cells also ultimately outgrew or destroyed their vasculature, but at a much larger relative tumor volume, conceivably a point at which angiogenic growth is no longer able to keep pace with rapid tumor cell proliferation.

With respect to virtually all pathophysiological indices studied, altered treatment sequencing had no measurable influence. Over two weeks of treatment, tumor growth rate was equivalent between schedules for both tumor models. In addition, at days 4, 7 and 11, total and perfused vessel spacing increased significantly with combination therapy, but no differences were found between schedules. Dissociation of both immature pericytes (PDGFR β +), basement membrane (type-IV collagen+), tumor hypoxia also increased progressively with treatment, but again with no dependence on schedule.

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